PA 277561

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

4

United States Patent and Trademark Office

CA00/00663

July 21, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/137,732***
FILING DATE: ***June 07, 1999***

RULE 17.1(a) OR (b)

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. SWAIN
Certifying Officer

1/PRT

PTO/SB/16(11-95)
Approved for use through 01/31/98 OMB 0651-0037
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

JC580 U.S. PTO 06/07/99

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(b)(2)

Docket Number	UBC 98-113	Type a plus sign (+) inside this box →	

JC541 U.S. PTO 60/137732 06/07/99

INVENTOR(s)/APPLICANT(s)			
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Moise	Alexandru	R.	Vancouver, Canada
Vitalis	Timothy	Z.	Vancouver, Canada
Jefferies	Wilfred	A.	Vancouver, Canada

TITLE OF THE INVENTION (280 characters max)

Method for the Evasion of Immune Response by Adenovirus E3/6.7K

CORRESPONDENCE ADDRESS

Maureen A. Beattie
C/o Industry Liaison
University of British Columbia
2194 Health Sciences Mall
IRC Bldg., Rm. 331, Vancouver Telephone (604) 822-8594

Province	British Columbia	Postal Code	V6T 1Z3	COUNTRY	Canada

ENCLOSED APPLICATION PARTS (check all that apply)

X	Specification	*Number of Pages*	14	X	Small Entity Statement
X	Drawing(s)/Figures(s)	*Number of Sheets*	12	☐	Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

X	A check or money order is enclosed to cover the filing fees	FILING FEE AMOUNT(S)	$75 00
☐	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number.		

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

X No

☐ Yes, the name of the U.S. government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE Maureen A. Beattie Date June 4/99

TYPED OR PRINT NAME Maureen A. Beattie REGISTRATION NO. (if appropriate)

☐ Additional inventors are being named on separately numbered sheets attached hereto.

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Burden Hour Statement: This form is estimated to take .2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SENT FEES OR COMPLETED FORMS TO THIS ADDRESS. SENT TO Box Provisional Application, Assistant Commissioner of Patents, Washington, DC 20231

THE UNIVERSITY OF BRITISH COLUMBIA

June 4, 1999.

Hon. Commissioner of Patents and Trademarks,
U.S. Patent and Trademark Office,
U.S. Department of Commerce,
Box Provisional Application,
Assistant Commissioner of Patents,
WASHINGTON, DC 20231,
U.S.A.

Dear Sir:

Re: Provisional Application for "Method for the Evasion of Immune Response by Adenovirus E3/6.7K" UBC file 98-113

Enclosed please find the necessary documents for filing a Provisional Application for the above-identified application on behalf of The University of British Columbia. Also enclosed is a cheque in the amount of $75.00 US being payment for the filing fee.

Thank you,

Sincerely,

Maureen A Beattie

Maureen A. Beattie,
Patent Administrator.

/mb
Encl.

U·I·L·O

UNIVERSITY-INDUSTRY
LIAISON OFFICE

IRC Room 331
2194 Health Sciences Mall
Vancouver, BC, Canada V6T 1Z3

Tel: (604) 822-8580
Fax: (604) 822-8589
Web: www.uilo.ubc.ca

TO:

Commissioner of Patents and Trademarks
Correspondence and Mail Division
Patents and Trademarks Office
Crystal Plaza
Building 2, Room 1A01
Arlington, Virginia
USA 22202

Enclosures:

1. Provisional application for patent cover sheet
2. Fee of $75.00 US
3. Declaration of nonprofit organization and Declaration of Independent Inventor
4. Specifications, 14 pages
5. Drawings/Figures (in support of specification) (12 pages)

METHOD FOR THE EVASION OF IMMUNE RESPONSE BY ADENOVIRUS E3/6.7K

BACKGROUND OF THE INVENTION

This application relates to a method for inhibiting apoptosis using Adenovirus E3/6.7K.

Viral infection is a cellular injury, and it results in the induction of programmed cell death of the host cell. Many viruses, particularly persistent DNA viruses modify the apoptotic response of a cell to allow continued virus replication. Apoptosis can be induced by the members of the TNF receptor super-family such as Fas (APO-1 or CD95) and p55 Tumor Necrosis Factor Receptor (p55 TNFR) as well as the death domain-containing receptors 3, 4 and 5 (DR3, DR4 and DR5, respectively). The intracellular factors responsible for death of the cell are highly conserved across species and are the target of viral inhibitors of apoptosis. It appears that proteins belonging to very different classes of virus have evolved to block the same cellular apoptotic event. This convergent evolution is evidenced by the classification of viral inhibitors of apoptosis. For example, adenovirus E1B 55K(Debbas and White, 1993), SV40 Large T antigen(Levine, 1997)(Lill et al., 1997) and human papilloma virus E6(Levine, 1997) inhibit p53-mediated lysis. The cellular survival factor Bcl-2 is mimicked by adenovirus E1B 19K(White, 1996), Epstein-Barr virus BHRF1(Henderson et al., 1993) and African swine fever virus LMW5-HL(Neilan et al., 1993). Members of the Interleukin 1b Converting Enzymes (ICE)-family of terminal proteolytic enzymes, also known as caspases, are blocked by baculovirus p35(Clem et al., 1991)(Xue and Horvitz, 1995) and crmA, the cowpox serpin protein(Zhou et al., 1997)(Tewari and Dixit, 1995). The adenovirus E3/10.4K and E3/14.5K downregulate surface Fas(Elsing and Burgert, 1998)(Tollefson et al., 1998)(Shisler et al., 1996), while the Inhibitors of Apoptosis (IAP) family of baculovirus and mammalian homologues interact with the TNF-α receptor associated factors (TRAFs) therefore blocking the signalling cascade that leads to the recruitment of caspases (Liston et al., 1996)(Duckett et al., 1996)(Deveraux et al., 1997). The activation of FADD-like interleukin-1beta-converting enzyme (FLICE), also known as caspase-8, through Fas is blocked by viral-FLICE-inhibitory proteins

(vFLIPs), found in the genomes of various types of herpesvirus(Thome et al., 1997) and by the E3/14.7K of adenovirus(Chen et al., 1998).

Adenovirus (Ad) is a very common human pathogen that results in persistent infections of the respiratory or gastrointestinal tract (Fox et al., 1969)(Fox et al., 1977). Persistent infections stem from an elaborate evasion of the host defense mechanisms. The adenovirus genes responsible for immune evasion map to the Early 3 (E3) region of the Ad genome (Wold and Gooding, 1989). The persistence, ease of infection and weak pathogenesis have made adenovirus suitable as vectors for gene therapy. Currently, Ad gene transfer vectors are the most efficient technique available for *in vivo* gene transduction. The size of the transduced DNA that can be accommodated by adenovirus is greater than 30kbp greatly surpassing all other viral systems. However, the major impediment for the success of Ad vectors as well as all the other gene transfer technologies is the unexpectedly strong immune response to infected target cells. In the case of Ad vectors the genetic makeup of the original vectors was designed to accommodate large fragments of DNA for the transduced gene, to the expense of areas of the adenoviral genome that were considered dispensable. The E3 region was one of the first areas to be replaced. This decision is now being questioned as it has been shown recently that the genes that made adenovirus a very persistent pathogen are encoded in the E3 region and are missing in the Ad vectors currently available. The strong immune response to Ad vectors is mediated by the circulating cytokine Tumor Necrosis Factor (TNF) α (Elkon et al., 1997) and by the innate immune response (Worgall et al., 1997). The negative effects of an immune response can be alleviated by implementing immunomodulatory proteins that allow the vector and the transduced cells to survive the immune response (Zhang et al., 1998). One such protein is E3/6.7K, a protein that allows the infected cell to evade the effects of TNF-α.

The E3/6.7K sequence does not have any significant homology to any other known proteins. It is well conserved between group C Ad2 and Ad5 adenovirus and between group B Ad3, Ad7 and Ad35, adenovirus(Hawkins et al., 1995). The Ad2 E3/6.7K protein(Wilson-Rawls et al., 1990) has been shown to be an integral membrane protein localized to the endoplasmic reticulum (ER)(Wilson-Rawls and Wold, 1993). The protein is present in two forms, one unglycosylated with an apparent molecular weight of 8kDa and one glycosylated with an apparent weight of 14kDa. The protein, though targeted to the ER, does not have a cleavable

signal sequence, but it has a hydrophobic central region that could act as a signal anchor(Wilson-Rawls et al., 1994).

The evasion of immune response is also a central impediment to the establisment of successful transplant technology as well as the treatment of autoimmune and neurodegenerative diseases. Apoptosis of the affected organ is often the result of neurodegenerative inflammatory disease. Factors that prevent apoptosis could lead to better therapies for these conditions.

Cell culture reactor expression systems are limited only by the ability of cells to grow and produce proteins of commercial or medical interest (Singh and al-Rubeai, 1998) (al-Rubeai, 1998). As cell grow they reach densities where protein production stops and producer cells undergo apoptosis in response to factors that are currently poorly characterized (al-Rubeai and Singh, 1998). There is potential for improving protein yield by avoiding the apoptotic response of cells grown in culture by including an antiapoptotic protein in the makeup of the cell (Simpson et al., 1998).

It is an object of the present invention to provide a method for immune evasion during neurodegenerative or inflammatory disease by implementing the E3/6.7K immunomodulatory protein from adenovirus. It is a further object of this invention to provide vectors containing the adenovirus E3/6.7K region for gene therapy or to minimize transplant rejection. It is a further object of this invention to provide a method for improving protein yield by avoiding the apoptotic response of cells grown in culture.

SUMMARY OF THE INVENTION

In accordance with the present invention a method for inhibiting apoptosis using adenovirus E3/6.7K is described. Adenovirus E3/6.7K protein prevents Tumor Necrosis Factor α (TNF-α) induced apoptosis in transfected cells. The gene coding for E3/6.7K is located in the E3 region of the adenovirus genome along with other proteins involved in evasion of the immune response. Both TNF-α induced apoptosis and TNF-α induced release of arachidonic acid are significantly reduced in U937 cells expressing E3/6.7K by stable transfection. The mechanism of E3/6.7K involves the cleavage and inactivation of cytosolic phospholipase A2 (cPLA2), this enzyme is a key player in the generation of proinflammatory agents. E3/6.7K has no sequence

homology to any of the previously described inhibitors of apoptosis, furthermore none of the previously described inhibitors of apoptosis localized specifically to the endoplasmic reticulum. E3/6.7K represents a new class of viral inhibitors of apoptosis localized to the endoplasmic reticulum.

The invention provides a method for immune evasion and evasion of apoptosis by implementing the E3/6.7K immunomodulatory protein from adenovirus.

The invention also provides vectors containing the adenovirus E3/6.7K region for gene therapy or alone to minimize transplant rejection. The invention also provides a method for improving protein yield from cell culture.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Shows viral titers of wild type virus (Ad5wt) and virus deleted for E3/6.7K(dl739) during the course of infection

Figure 2. Shows pathology and histologic scoring of animals infected with Ad5wt or dl739 at various times post infection (p.i.), showing bronchiolar and perivascular inflammatory cell infiltration.

Figure 3. Shows tricine SDS-PAGE Analysis of immunoprecipitated E3/6.7K. U937 cells transfected with vector alone (lane 1) or transfected with E3/6.7K cDNA.

Figure 4. Shows analysis of the effect of E3/6.7K on the inducible release of radiolabelled arachidonic acid.

Figure 5. Shows annexin V-FITC flow cytometry analysis of apoptosis induced cells.

Figure 6. Shows immunoblotting of cPLA2 indicating the cleavage and inactivation of this enzyme in the presence of E3/6.7K.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for evading a hosts immune response to improve the delivery of genes or peptides and for minimizing transplant rejection. The invention

also provides a method for improving protein yield from cell culture. The invention will now be described with reference to the following non-limiting examples.

Virus strains and tissue culture. Wild Type Ad5 (Ad5wt) was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and dl739, E3/6.7K-deleted viral mutant (dl739)(Brady et al., 1992) was obtained as a gift from W.S.M Wold. These two Adenovirus group C viruses share a great degree of similarity, but differ in the expression of E3/6.7K protein, which is deleted in dl739 as described previously(Brady et al., 1992). Both viral serotypes were propagated in monolayer culture of A549 cells grown in Minimal Essential Media (Gibco BRL Life Technologies Inc., Gaithersburg, Maryland, USA) supplemented with 10% Fetal Calf Serum (FCS). Two to five days after inoculation with Ad5, cells were freeze/ thawed twice, sonicated for 30s three times and centrifuged at 500xg for 5 min. The supernatant was collected and its viral titer determined by plaque assays on A549 monolayers grown on six well plates. Titers ranged from 10^8 to 10^9 plaque-forming units (pfu)/ml. Control inoculum was prepared from uninfected A549 cells treated in an identical manner to the infected cells.

Inoculation of airway ducts and viral plaque assays. Two groups of 24 mice were anaesthetized with Halothane. One group of mice were infected intranasally with 10^7pfu of Ad5wt in 60 µl of culture media while the other group of mice was infected intranasally with 10^7pfu of dl739 in 60 µl of culture media. In addition, six animals were infected with sterile culture media alone. Six animals from each of the two groups were sacrificed with an overdose of Halothane 2 hours, 1, 3 and 7 days post infection (p.i.). Two sham infected animals were sacrificed on days 1, 3 and 7 days p.i.. The left lung was removed and frozen in liquid nitrogen for use in viral plaque assays. The right lung was inflated with 4% paraformaldehyde in PBS pH7.4 (0.149 M NaCl, 0.012 M Na2HPO4, 0.004M KH2PO4) and embedded in paraffin.

Viral titer. Viral plaque assays were used to quantitate the amount of replicating virus in mouse lungs. Approximately 200mg of lung was homogenized on ice in 1ml sterile MEM with a polytron. The homogenate was spun for 2 min at 10,000g while the supernatant was removed and stored at -70°C. The lung homogenate supernatant titer was determined by plaque assay on A549 cell monolayer cultures grown in MEM/10%FBS on six well plates using decimal dilutions from 10-1 to 10-6 in MEM from the supernatant of animal lung homogenate from all time groups. Each well was inoculated with 500 µl of diluted supernatant and virus was allowed to adsorb

onto the monolayer of A549 cells for 1h. at 37°C An agarose overlay (0.9% agarose, MEM, 2% FCS, and 0.001 neutral red at 37°C) was applied after adsorption. Plaques were counted after 10-14 days and normalized to lung mass and expressed as (log pfu/g lung tissue).

Histologic scoring. Four μm sections of paraffin embedded lung tissue were mounted on glass slides and stained with hematoxylin and eosin. An independent observer, unaware of the experimental treatment of the tissue sections, scored the airway mucosal, airway adventitia and the vascular adventitia for inflammation. The histopathologic grades were 0 - no inflammation, 1 – mild inflammation. 2 – moderate inflammation, 3 – severe inflammation for each feature. The scores for each feature were summed to give a total inflammatory score with maximum being 9 for each animal. A mean inflammatory score was calculated for each animal by dividing the total score by 3. The mean and standard deviation was calculated for each experimental group.

Statistical analysis. Comparisons between the two virus were made for viral titer, inflammatory score and time using a 2-way ANOVA. The level of significance was $p<0.05$.

Plasmid constructs. The cDNA for E3/6.7K has been obtained by amplifying by PCR the region coding for the E3/6.7K ORF from a vector carrying the Ad2 E3 region (obtained as a gift from W.S.M. Wold). The PCR product was cloned in the Xho1 site of the BPV based cDNA expression vector pBCMGSneo(Karasuyama and Melchers, 1988) and sequenced to insure accuracy.

Generation of stable U937 cell lines expressing E3/6.7K. U937 human histiocytic lymphoma cells (Sundstrom and Nilsson, 1976) obtained from ATCC (CRL 1593) were maintained in RPMI 1640, 10% FCS, 2mM L-glutamine, 10mM HEPES, 100U/ml penicillin and 100μg/ml streptomycin in an atmosphere of 5% CO2 and 100% humidity. Cells were transfected with the appropriate construct by using the DMRIE-C cationic lipid reagent available from Life Technologies using the manufacturer's protocol. Transfected cells were maintained in medium containing geneticin G-418 sulphate at a final concentration of 800μg/ml. Media and supplements were purchased from Life Technologies. Subclones of the transfected cell lines were generated by serial dilution and examined for expression of E3/6.7K by Northern Blotting. The expression of E3/6.7K mRNA was very similar in all the clones examined. All the G-418 resistant cells that survived the selection procedure were pooled and used for the in vitro assays, in order to avoid clonal variations known to arise in U937 cells.

Labelling, immunoprecipitation and Western Blotting of proteins from transfected cells. U937 cells transfected with vector or with vector carrying E3/6.7K were grown in suspension until they were growing exponentially. 10^8 cells were harvested, washed and intracellular pools of cysteine and methionine were depleted by incubation in prewarmed methionine/cysteine-free essential media without FCS for one hour at 37°C at a concentration of $5x10^6$ cells/ml. A total of $2x10^7$ cells were labelled for one hour in prewarmed methionine/cysteine-free media containing 0.5mCi/ml [35S]-Cysteine and 0.2 mCi/ml (Amersham) [35S]-Methionine (Amersham) at a concentration of $5x10^6$ cells /ml. Cells were washed and then lysed on ice in freshly made lysis buffer containing 1% TritonX-100, 1% BSA (bovine serum albumin), 1mM iodoacetamide, 1mM PMSF, 2.5TIU/ml aprotinin, 0.01M Tris pH8.0, 0.14M NaCl. Samples were counted by TCA precipitation and approx. 10^7cpm of each sample was precleared O/N using protein A-Sepharose CL-4B, the supernatant was immunoprecipitated using a polyclonal rabbit antiserum raised against the C-terminal portion of E3/6.7K and protein A-Sepharose. The pellet was denatured in SDS/sample buffer and loaded on a Tricine-SDS PAGE gel, 16.5%T, 3%C separating gel with a 10%T, 3%C spacer gel (Schagger and von Jagow, 1987). Alternatively, cell lysate equivalent to 105 cells was denatured in SDS-PAGE loading buffer and loaded on 10% glycine SDS-PAGE gel system, separated and blotted onto a Immobilon-P PVDF membrane (Millipore) and probed with cPLA2 rabbit polyclonal antiserum (Cayman Chemical). The signal was detected via horse radish peroxidase-conjugated, goat antirabbit antiserum and by chemiluminescence using the ECL kit (Biorad).

Arachidonic acid release assays. Cells were grown at low density in 10% Hyclone FCS, RPMI 1640, 2mM L-glutamine, 10mM HEPES for several days then harvested and washed twice in PBS, 1% BSA. Approximately $5x10^6$ cells ($5x10^5$ cells/ml) were labelled for 20hrs in same media as above supplemented with 0.4 μCi/ml [3H]arachidonic acid [5,6,8,9,11,12,14,15-3H(N)] (0.1mCi/ml stock; New England Nuclear). Cells were washed twice in RPMI 1640, 0.2%BSA and incubated for one hour in the wash media in order to minimize the spontaneous release of [3H]arachidonic acid. The number of cells was normalized in all cell lines and 400μl of cell suspension was aliquoted in each well of a 24 well plate containing 100μl of treatment media ($2x10^5$ cells/well corresponding to $1.4x10^3$ counts/well). The assay was set up in triplicate and the cells were stimulated either with media alone or with 20ng/ml human rTNF-α (2000U/ml)

(Boehringer, Mannheim), or with 10μg/ml cycloheximide or with a combination of 20ng/ml TNF-α and 10μg/ml cycloheximide. After 20 hours of treatment the cells were centrifuged and 100μl of supernatant out of 500μl total was mixed with 3ml scintillation fluid and counted. For each cell line three samples were lysed in lysis buffer and the lysate was used to determine the total counts of incorporated [3H]Arachidonic Acid. The counts per minute of released [3H]arachidonic acid were expressed as a percentage of the average of total incorporated [3H]arachidonic acid.

Annexin V-FACS apoptosis assay. Annexin V-FITC (PharMingen) was used to determine the binding of Annexin V to externalized phosphatidyl serine. The protocol followed was based on the manufacturers Annexin V-FITC staining protocol. Cells were grown at low density in 10% Hyclone FCS, RPMI 1640, 2mM L-glutamine, 10mM HEPES for several days then $5x10^6$ cells were harvested and washed twice in PBS. Cells resuspended in above media were treated for 7 hours with media alone or with 100ng/ml (10,000 U/ml) human rTNF-α or with 200 μg/ml cycloheximide or with a combination of 100 ng/ml TNF-α and 200 μg/ml cycloheximide. The cells were resuspended at $1x10^6$ cells/ml in 1xBinding Buffer (10mMHepes/NaOH, pH7.4, 140mM NaCl, 2.5mM CaCl2). $1x10^5$ cells (100μl of above suspension) were combined with 5μl of Annexin V-FITC. One sample of cells was not stained and used to set up the baseline fluorescence. We examined the cells with a fluorescence-activated cell sorter (FACS) on a Beckton Dickson FACS Analyzer.

Production of Ad vectors for gene therapy. The backbone to be used for gene therapy is based on the SV5 backbone previously described (Chen 1997 PNAS). This backbone has been sucessfully used to transduce in vivo the dystrophin gene. The backbone lacks the E1 and E2 region. Without these two regions the SV5 Ad vector is replication defective and therefore safer to use as well as it elicits a reduced inflammatory response. The cDNA encoding E3/6.7K under the control of the actin promoter and the CMV enhancer is added to SV5 and used to rescue a new vector called SV5-6.7, which will incorporate E3/6.7K as an immunomodulatory protein.

Creation of Producer Cells resistant to apoptosis. The creation of hybridoma, chinese hamster ovary (CHO) or insect cells that are resisitant to apoptosis will follow the same procedure as the transfection of U937 cells outlined with the following exception. At the end of

the selection in G-418 the cells will be sorted or clonally expanded in order to screen for the expression of the protein of interest.

EXAMPLES

E3/6.7K Results in more Persistent Viral Titers and a reduction of the inflammatory response. We found that the presence of E3/6.7K results in more persistent viral titers during the course of infection by comparing mice infected with a E3/6.7K deletion virus (dl739)(Brady et al., 1992) with mice infected with the wild type virus (Ad5wt) (fig. 1). The titers of dl739 (E3/6.7K deleted) were significantly higher than Ad5 wild type (Ad5wt) one day after inoculation ($p<0.001$, fig. 1). Over time the titers of dl739 decreased as the virus was cleared ($p<0.001$). In contrast Ad5wt titers did not change significantly over the 7 day experimental period. We attribute the rapid reduction of dl739 over the seven days period to a strong host response due to the increased inflammation in the absence of E3/6.7K. Inflammation of the perivascular region of the blood vessels and the adventitia of the airways was greater in animals infected with dl739 than in animals infected with Ad5wt over the seven days experimental period ($p=0.025$, fig. 2). There was also a significant increase in inflammation from day three to day seven for both types of viruses ($p=0.029$).

TNF-α Mediated Arachidonic Acid Release Is Reduced in the Presence of E3/6.7K. We investigated whether E3/6.7K can affect the cellular reponse to inflammatory cytokines. We created a U937 cell line transfected with the cDNA for E3/6.7K and we confirmed that the E3/6.7K was expressed using immunoprecipitation with a polyclonal rabbit antiserum raised against an E3/6.7K C-terminal derived peptide and SDS-PAGE electrophoresis (fig 3). The U937 cells transfected with E3/6.7K cDNA (U937-E3/6.7K) decreased [3H]arachidonic acid release by 50% when compared with U937 cells transfected with vector alone (U937neor) when stimulated with TNF-α (fig. 4). When the stimulus was increased by the addition of TNF-α and cycloheximide (CHX), a protein synthesis inhibitor synergistic with TNF-α, U937-E3/6.7K were still able to reduce the release of [3H]arachidonic acid by 60% when compared to U937neor. The presence of E3/6.7K reduces the levels of inducible release of [3H]arachidonic acid during TNF-α stimulation.

Apoptosis Induced by TNF-α is Reduced in the Presence of E3/6.7K. We assayed TNF-a induced apoptosis by measuring the externalization of phosphatidyl serine by using FITC labelled Annexin V(Martin et al., 1995). Cells expressing E3/6.7K show a 55% reduction in percentage of apoptotic cells compared with U937neor following stimulation with TNF-α (fig. 5). The U937-E3/6.7K cells show a 65% reduction in apoptosis compared to U937neor following an augmented stimulation with a combination of TNF-α and CHX. The presence of E3/6.7K results decreased the apoptotic response in U937 cells upon stimulation with TNF-α or a combination of TNF-α and CHX.

In the Presence of E3/6.7K, cPLA2 Is Cleaved to a 78kDa Form Following TNF-α Induction. We assayed the expression of cPLA2 in U937 cells following induction with TNF-α. The cPLA2 antiserum recognized two forms of the enzyme one larger form of approximately 110kDa and a second form of 78kda (fig. 6). There is a noticeable difference between U937neor cells and U937-E3/6.7K with regards to the ratio of the 110kDa versus the 78kDa forms of cPLA2. While TNF-α does not seem to alter this ratio in U937neor, cells where the predominant form migrates as a 110kDa protein, in U937-E3/6.7 K cells following induction with TNF-α the most predominant form of cPLA2 is 78kDa. The antisera used in our study was raised against a peptide corresponding to residues 443-462 of the cPLA2 sequence, therefore the only fragment detected by immunoblotting following cleavage is the 78kDa fragment corresponding to the 1-522 amino acid sequence of cPLA2 as isolated from U937 cells (Sharp et al., 1991).

References

al-Rubeai, M. (1998) *Adv Biochem Eng Biotechnol,* **59,** 225-249.

al-Rubeai, M. and Singh, R. P. (1998) *Curr Opin Biotechnol,* **9,** 152-156.

Brady, H. A., Scaria, A. and Wold, W. S. (1992) *J Virol,* **66,** 5914-5923.

Chen, P., Tian, J., Kovesdi, I. and Bruder, J. T. (1998) *J Biol Chem,* **273,** 5815-5820.

Clem, R. J., Fechheimer, M. and Miller, L. K. (1991) *Science,* **254,** 1388-1390.

Debbas, M. and White, E. (1993) *Genes Dev,* **7,** 546-554.

Deveraux, Q. L., Takahashi, R., Salvesen, G. S. and Reed, J. C. (1997) *Nature,* **388,** 300-304.

Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dongen, J. L., Gilfillan, M. C., Shiels, H., Hardwick, J. M. and Thompson, C. B. (1996) *Embo J,* **15,** 2685-2694.

Elkon, K. B., Liu, C. C., Gall, J. G., Trevejo, J., Marino, M. W., Abrahamsen, K. A., Song, X., Zhou, J. L., Old, L. J., Crystal, R. G. and Falck-Pedersen, E. (1997) *Proc Natl Acad Sci U S A,* **94,** 9814-9819.

Elsing, A. and Burgert, H. G. (1998) *Proc Natl Acad Sci U S A,* **95,** 10072-10077.

Fox, J. P., Brandt, C. D., Wassermann, F. E., Hall, C. E., Spigland, I., Kogon, A. and Elveback, L. R. (1969) *Am J Epidemiol,* **89,** 25-50.

Fox, J. P., Hall, C. E. and Cooney, M. K. (1977) *Am J Epidemiol,* **105,** 362-386.

Hawkins, L. K., Wilson-Rawls, J. and Wold, W. S. (1995) *J Virol,* **69,** 4292-4298.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G. and Rickinson, A. (1993) *Proc Natl Acad Sci U S A,* **90,** 8479-8483.

Karasuyama, H. and Melchers, F. (1988) *Eur J Immunol,* **18,** 97-104.

Levine, A. J. (1997) *Cell,* **88,** 323-331.

Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J. and Livingston, D. M. (1997) *Nature,* **387,** 823-827.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A. and Korneluk, R. G. (1996) *Nature,* **379,** 349-353.

Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M. and Green, D. R. (1995) *J Exp Med,* **182,** 1545-1556.

Neilan, J. G., Lu, Z., Afonso, C. L., Kutish, G. F., Sussman, M. D. and Rock, D. L. (1993) *J Virol,* **67,** 4391-4394.

Schagger, H. and von Jagow, G. (1987) *Anal Biochem,* **166,** 368-379.

Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R. and et al. (1991) *J Biol Chem,* **266,** 14850-14853.

Shisler, J., Duerksen-Hughes, P., Hermiston, T. M., Wold, W. S. and Gooding, L. R. (1996) *J Virol,* **70,** 68-77.

Simpson, N. H., Singh, R. P., Perani, A., Goldenzon, C. and Al-Rubeai, M. (1998) *Biotechnol Bioeng,* **59,** 90-98.

Singh, R. P. and al-Rubeai, M. (1998) *Adv Biochem Eng Biotechnol,* **62,** 167-184.

Sundstrom, C. and Nilsson, K. (1976) *Int J Cancer,* **17,** 565-577.

Tewari, M. and Dixit, V. M. (1995) *J Biol Chem,* **270,** 3255-3260.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroter, M., Scaffidi, C., Krammer, P. H., Peter, M. E. and Tschopp, J. (1997) *Nature,* **386,** 517-521.

Tollefson, A. E., Hermiston, T. W., Lichtenstein, D. L., Colle, C. F., Tripp, R. A., Dimitrov, T., Toth, K., Wells, C. E., Doherty, P. C. and Wold, W. S. (1998) *Nature,* **392,** 726-730.

White, E. (1996) *Genes Dev,* **10,** 1-15.

Wilson-Rawls, J., Deutscher, S. L. and Wold, W. S. (1994) *Virology,* **201,** 66-76.

Wilson-Rawls, J., Saha, S. K., Krajcsi, P., Tollefson, A. E., Gooding, L. R. and Wold, W. S. (1990) *Virology,* **178,** 204-212.

Wilson-Rawls, J. and Wold, W. S. (1993) *Virology,* **195,** 6-15.

Wold, W. S. and Gooding, L. R. (1989) *Mol Biol Med,* **6,** 433-452.

Worgall, S., Wolff, G., Falck-Pedersen, E. and Crystal, R. G. (1997) *Hum Gene Ther,* **8,** 37-44.

Xue, D. and Horvitz, H. R. (1995) *Nature,* **377,** 248-251.

Zhang, H. G., Zhou, T., Yang, P., Edwards, C. K., 3rd, Curiel, D. T. and Mountz, J. D. (1998) *Hum Gene Ther,* **9,** 1875-1884.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M. and Salvesen, G. S. (1997) *J Biol Chem,* **272,** 7797-7800.

What is claimed is:

1. A method for inhibiting apoptosis of a cell comprising treating the cell with an effective amount of the protein encoded by E3/6.7K (6.7).

2. The method of claim 1 wherein the treating step comprises administering to the cell a polynucleotide encoding 6.7 and wherein 6.7 is expressed in the cell.

3. The method of claim 2 wherein the polynucleotide comprises a recombinant adenovirus vector.

4. The method of claim 1 wherein the cell comprises a transplant tissue.

5. The method of claim 1 wherein the cell comprises an eukaryotic cell culture expression system.

6. The method of claim 1 wherein the treating step comprises administering 6.7 to the cell.

7. The method of claim 6 wherein 6.7 is administered with a carrier which facilitates delivery of 6.7 into the cell.

8. A method for decreasing apoptosis of target cells in a patient comprising treating the patient with an effective amount the protein produced by E3/6.7.

9. The method of claim 8 wherein the treating step comprises administering to the patient a polynucleotide encoding 6.7 and wherein the polynucleotide is internalized in the target cells and 6.7 is expressed.

10. The method of claim 9 wherein the polynucleotide comprises a recombinant adenovirus vector.

11. The method of claim 2 wherein the cells being administered are used in a cell culture expression system.

12. The method of claim 8 wherein the patient suffers from a degenerative disease or an immunodeficiency disease.

13. The method of claim 8 wherein the treating step comprises administering a polynucleotide encoding 6.7 and wherein 6.7 is expressed in the patient.

14. The method of claim 13 wherein the polynucleotide comprises a recombinant adenovirus vector.

15. The method of claim 13 wherein the polynucleotide comprises a recombinant virus vector.

16. The method of claim 13 wherein 6.7 is administered with a carrier which facilitates delivery of 6.7 into the cells.

17. A method of decreasing leukocyte apoptosis in a patient comprising:
(a) withdrawing leukocytes from the patient, (b) treating the leukocytes with an effective amount of a 6.7, and (c) administering the treated leukocytes to the patient.

18. The method of claim 17 wherein the treating step comprises administering to the leukocytes a polynucleotide encoding 6.7 wherein 6.7 is expressed in the leukocytes.

19. The method of claim 18 wherein the polynucleotide comprises a recombinant adenovirus vector.

20. A composition comprising a protein produced by E3/6.7 and a carrier suitable for facilitating delivery of 6.7 into a cell.

21. A recombinant adenovirus comprising a polynucleotide encoding the protein produced by E3/6.7 complex operably linked to a promoter, wherein the adenovirus is replication defective and wherein the polynucleotide is expressed upon infection of a eukaryotic cell with the adenovirus.

22. A recombinant viral vector comprising a polynucleotide encoding the protein produced by E3/6.7 complex operably linked to a promoter, wherein the virus is replication defective and wherein the polynucleotide is expressed upon infection of a eukaryotic cell with the virus.

23. The method of claim 2 wherein the polynucleotide comprises a recombinant virus vector.

24. The method of claim 18 wherein the polynucleotide comprises a recombinant virus vector.

25. The method of claim 8 wherein the patient suffers from an inflammatory disease or a neurodegenerative disease.

26. The method of claim 9 wherein the polynucleotide comprises a recombinant virus vector.

Applicant or Patentee: **Alexandru R. Moise, Timothy Z. Vitalis and Wilfred A. Jefferies**

Serial or Patent No.:

Filed or Issued:

For: **Method for the Evasion of Immune Response by Adenovirus E3/6.7K**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9 (f) and 1.27 (b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9 (c) for purposes of paying reduced fees under section 41 (a) and (b) of Title 35, United States Code to the Patent and Trademark Office with regard to the invention entitled:

Method for the Evasion of Immune Response by Adenovirus E3/6.7K

described in

[X] the specification filed herewith
[ ] Application Serial No.
[ ] Patent no. issued

I have not assigned, granted, conveyed or licensed and am under not obligation, under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9 (c) if that person made an invention or to any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

Each person, concern or organization to which I have assigned, granted, conveyed or licensed or am under an obligation under contract or law to assign, grant, convey or license any right in the invention is listed below:

[ ] no such person , concern or organization.
[X] persons, concerns or organizations listed below

FULL NAME **The University of British Columbia**

ADDRESS **2075 Wesbrook Mall, Vancouver, British Columbia, Canada V6T 1Z1**

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b)). I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or

imprisonment, or both under section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which the verified statement is directed.

Alexandru R. Moise
NAME OF INVENTOR

_____ _____
Signature of Inventor Date

Timothy Z. Vitalis
NAME OF INVENTOR

T. Vitalis 3 June, 1999
Signature of Inventor Date

Wilfred A. Jefferies
NAME OF INVENTOR

3 June, 1999
Signature of Inventor Date

UBC - 98-113

Applicant or Patentee: **Alexandru R. Moise, Timothy Z. Vitalis and Wilfred A. Jefferies**

Serial or Patent No.:

Filed or Issued:

For: **Method for the Evasion of Immune Response by Adenovirus E3/6.7K**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9 (f) and 1.27 (D)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below

NAME OF ORGANIZATION **The University of British Columbia**
ADDRESS OF ORGANIZATION **2075 Wesbrook Mall Vancouver, British Columbia, Canada V6T 1Z1**

TYPE OF ORGANIZATION [X] University or other institute of higher education

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9 (e) for purposes of paying reduced fees under section 41 (a) and (b) of Title 35, United States Code with regard to the invention entitled: **Method for the Evasion of Immune Response by Adenovirus E3/6.7K** inventors **Alexandru R. Moise, Timothy Z. Vitalis and Wilfred A. Jefferies.**

[X] the specification filed herewith
[ ] application serial no. , filed

I hereby declare that the rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b)).

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both under section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which the verified statement is directed.

NAME OF PERSON SIGNING Richard D. Spratley

Title of Person signing Director, Research Services

Address of Person Signing IRC Room 331 - 2194 HEALTH SCIENCES MALL
VANCOUVER, B.C V6T 1Z3

Signature _____ Date _____ 1997.9

Figure 1. Viral titers of wild type virus (Ad5wt) and virus deleted for E3/6.7K(dl739) during the course of infection. Virus cultured from the two groups of animals at 2 hours post infection (p.i.) indicate the same amount of virus was present at the time of inoculum. Days 1, 3 and 7 p.i. are represented in the above graph. Six animals from each group, Ad5wt and dl739 infected animals, were sacrificed on the given date and the isolated lung tissue was assayed through plaque assay for the presence of infectious viral particles. The viral titer is expressed as (plaque forming units)/gram of lung tissue isolated.

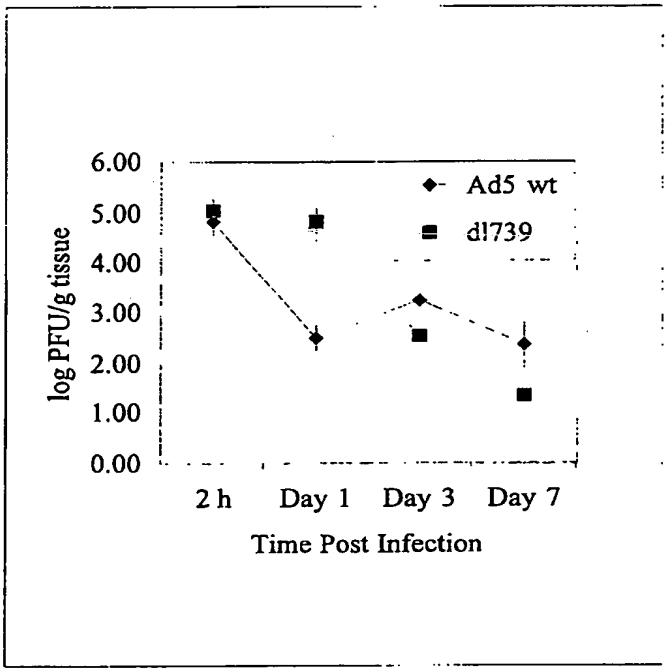
6.00
5.00
4.00
3.00
2.00
1.00
0.00
log PFU/g tissue
Ad5 wt
dl739
2 h
Day 1
Day 3
Day 7
Time Post Infection

FIG. 1

Figure 2 Pathology and histologic scoring of animals infected with Ad5wt or dl739 at various times post infection (p.i.), showing bronchiolar and perivascular inflammatory cell infiltration. A) Ad5wt sham infected, C) Ad5wt day 1p.i., E) Ad5wt day 3 p.i., G) Ad5wt day 7 p.i., B) dl739 sham infected, D) dl739 day 1 p.i., F) dl739 day 3p.i., G) dl739 day 7 p.i.

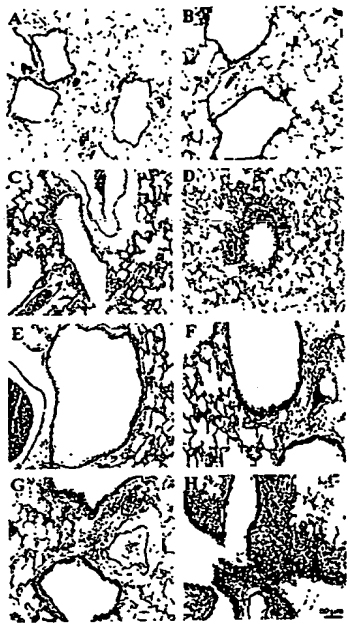

FIG. 2

Figure 3 Tricine SDS-PAGE Analysis of Immunoprecipitated E3/6.7K. U937 cells transfected with vector alone (lane 1) or transfected with E3/6.7K cDNA (lane 2) were radiolabelled with [35S]-Cysteine and [35S]-Methionine, lysed and immunoprecipitated with a polyclonal rabbit antiserum generated against the C terminal region of E3/6.7K and analyzed by Tricine SDS-PAGE protocol on a 16.5%T,3%C separating gel with a 10%T, 3%C spacer gel.

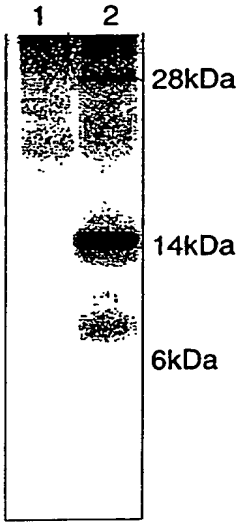

FIG. 3

Figure 4. Analysis of the effect of E3/6.7K on the inducible release of radiolabelled Arachidonic Acid. The assay was set up in triplicate and the cells were stimulated either with media alone or with 90ng/ml TNF-α, or with a 2μg/ml cycloheximide(CHX) or with a combination of 90ng/ml TNF-α and 2μg/ml cycloheximide. The counts per minute of released [^{3}H]Arachidonic Acid were expressed as a percentage of the average of total incorporated [^{3}H]Arachidonic Acid. The experiment was repeated three times with similar results.

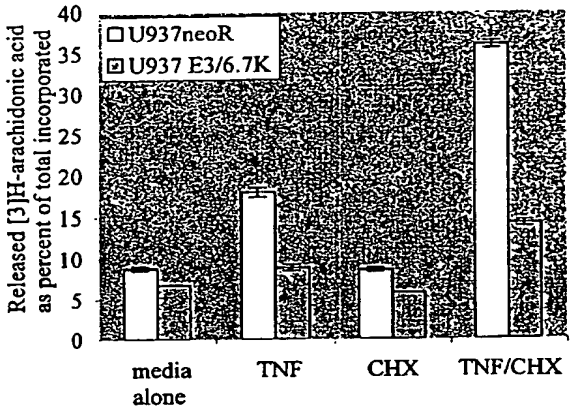
Released [3]H-arachidonic acid as percent of total incorporated
40
35
30
25
20
15
10
5
0
U937neoR
U937 E3/6.7K
media alone
TNF
CHX
TNF/CHX

FIG. 4

Figure 5 Annexin V-FITC Flow Cytometry Analysis of Apoptosis Induced Cells. Cells transfected with vector alone panels a, b, c, d or cells transfected with vector carrying E3/6.7K e, f, g, h were stimulated for 7 hours with a and e media alone, b and c 100ng/ml TNF-α, c and g 10μg/ml cycloheximide, d and h combination of 100ng/ml TNF-α and 10μg/ml cycloheximide. A sample of each cell line and treatment was stained with Annexin V-FITC and analyzed by FACS. A second sample was analyzed by FACS in the absence of Annexin V-FITC in order to determine the background fluorescence which corresponds to the fluorescence associated with the Annexin V negative cell population from each sample. The percentage of Annexin V positive, apoptotic cells is indicated for each sample in the FACS window. The experiment was repeated with similar results.

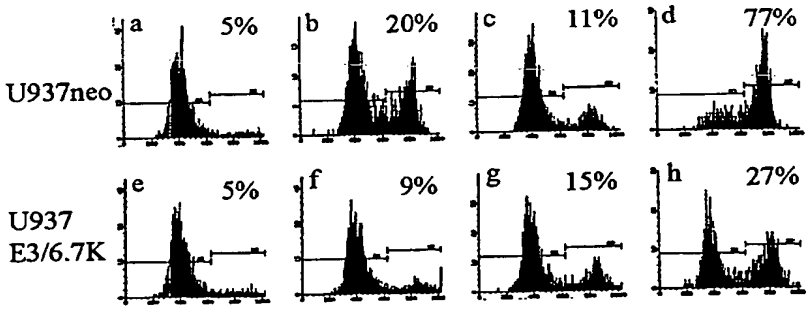

FIG. 5

Figure 6. Immunoblotting of cPLA2 indicating the cleavage and inactivation of this enzyme in the presence of E3/6.K. U937neo^r and U937-E3/6.7K cells were treated with media or with 50ng/ml TNF-α and 1μg/ml CHX for 6 hours. Cell lysates were immunoblotted with anti-cPLA$_2$ antisera recognizing the residues 443-462 in the sequence of the protein.

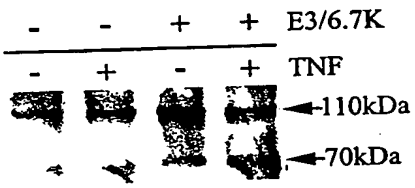

FIG. 6